The widely utilized brominated flame retardant tetrabromobisphenol A (TBBPA) is a potent inhibitor of the SERCA Ca^{2+} pump

Oluseye A. OGUNBAYO and Francesco MICHELANGELI[1]

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

TBBPA (tetrabromobisphenol A) is currently the most widely used type of BFR (brominated flame retardant) employed to reduce the combustibility of a large variety of electronic and other manufactured products. Recent studies have indicated that BFRs, including TBBPA, are bio-accumulating within animal and humans. BFRs including TBBPA have also been shown to be cytotoxic and potentially endocrine-disrupting to a variety of cells in culture. Furthermore, TBBPA has specifically been shown to cause disruption of Ca^{2+} homoeostasis within cells, which may be the underlying cause of its cytotoxicity. In this study, we have demonstrated that TBBPA is a potent non-isoform-specific inhibitor of the SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) (apparent K_i 0.46–2.3 μM), thus we propose that TBBPA inhibition of SERCA contributes in some degree to Ca^{2+} signalling disruption. TBBPA binds directly to the SERCA without the need to partition into the phospholipid bilayer. From activity results and Ca^{2+}-induced conformational results, it appears that the major effect of TBBPA is to decrease the SERCA affinity for Ca^{2+} (increasing the K_d from approx. 1 μM to 30 μM in the presence of 10 μM TBBPA). Low concentrations of TBBPA can quench the tryptophan fluorescence of the SERCA and this quenching can be reversed by BHQ [2,5-di-(t-butyl)-1,4-hydroquinone] and 4-*n*-nonylphenol, but not thapsigargin, indicating that TBBPA and BHQ may be binding to similar regions in the SERCA.

Key words: affinity, brominated flame retardant, calcium signalling, E1 and E2 conformation, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), tetrabromobisphenol A.

INTRODUCTION

Ca^{2+} plays an important role in many cellular signalling processes. Indeed, inadequate or prolonged elevation of intracellular Ca^{2+} levels may lead to deleterious effects, ranging from failure in signal transduction to apoptotic processes within and between cells [1]. Therefore, Ca^{2+} homoeostasis in the cytosol is very crucial to cell viability and function, with the potential for serious consequences arising when normal control is disturbed. The SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) has long been implicated as playing a central role in the mechanism of Ca^{2+} transport across the membrane, from the cell cytosol into the endoplasmic reticulum and this is one of the major mechanisms by which low levels of free cytosolic Ca^{2+} concentration are maintained in the cell. SERCA Ca^{2+} pumps belong to the P-type family of ion transporters [2] and three isoforms of this pump are known to exist which have a tissue-specific distribution [3]. The activities of SERCA Ca^{2+} pumps have been found to be altered by a wide spectrum of lipophilic molecules, including a number of environmental endocrine disrupters [4–6]. Although a number of these molecules have non-specific effects on these pumps, some of these hydrophobic molecules interact specifically at distinct sites on the protein and, in some cases, these molecules have proved invaluable in elucidating mechanistic steps within the Ca^{2+} transport processes [7–10].

TBBPA (tetrabromobisphenol A) is a highly lipophilic halogenated aromatic molecule and currently the most widely used type of BFR (brominated flame retardant) [11]. TBBPA and other BFRs are employed as additives in the manufacture of printed circuit boards and polymers used to make electrical equipment and textiles, in order to reduce the risk of combustion [11]. Owing to their widespread use and abundance, recent studies have shown that BFRs exist in the environment far from their location of production and/or use; and that the concentrations of some of the BFRs, both in the environment and in humans, are rapidly increasing [12]. Concentrations of various BFRs have been found at levels as high as 1 mg/kg of dried sewage slurry [13] and up to 160 ng/g in some samples of human serum lipid (equivalent to approx. 0.3 μM [14]). More specifically, TBBPA was found to be present at concentrations up to 1.8 ng/g of lipid in serum samples from individuals who dismantle electronic components [15].

In vitro studies have demonstrated that TBBPA is toxic to a variety of mammalian cell lines [16], as well as primary cells including hepatocytes [17], splenocytes [18] and cerebellar granule cells [19,20]. In addition, TBBPA also shows oestrogenic activity since it enhances the oestrogen-dependent proliferation of MtT/E-2 cells [21]. Furthermore, TBBPA at low micromolar concentrations has been shown to modulate a number of cell signalling processes such as the activities of tyrosine kinase, MAP (mitogen-activated protein) kinases, protein kinase C, as well as elevating the concentration of reactive oxygen species and cytosolic Ca^{2+} when added to neutrophils [22]. Also, a recent study has shown that low concentrations of TBBPA can increase cytosolic Ca^{2+} levels in cerebellar granule cells, leading to cell death [20].

The widespread production and use of BFRs and the evidence of increased contamination of the environment and organisms by these chemicals, coupled with the fact that they are cytotoxic to a variety of cells, has led us to investigate the effects of TBBPA, the most commonly used BFR, on SERCA Ca^{2+} pumps as a mechanism of disrupting Ca^{2+} signalling pathways within cultured cells.

Abbreviations used: BFR, brominated flame retardant; BHQ, 2,5-di-(t-butyl)-1,4-hydroquinone; CPA, cyclopiazonic acid; DOPC, dioleylphosphatidylcholine; PDA, 12-(1-pyrene)dodecanoic acid; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TBBPA, tetrabromo-bisphenol A; TCA, trichloroacetic acid.

[1] To whom correspondence should be addressed (email f.michelangeli@bham.ac.uk)

MATERIALS AND METHODS

TBBPA was purchased from Acros Organics (purity given as 97 %). All other reagents were of analytical grade. TBBPA was dissolved in dimethyl sulfoxide to give a stock solution of 20 mM and the solvent was never more than 1.0 % (v/ v) in the assays performed. The pK_a of TBBPA was determined in aqueous solution by the method described in [8]. Native SR (sarcoplasmic reticulum) was prepared from rabbit skeletal muscle as described in [23] and typically had a purity of approx. 80 % SERCA. Porcine cerebellar microsomes were prepared as described in [24].

SERCA activity

Measurement of SERCA activity in cerebellar microsomes was performed using the phosphate liberation assay as described in [25]. The effects of TBBPA on SERCA activity were investigated as a function of Ca^{2+} and ATP concentrations at different pH values, employing a coupled enzyme assay as previously described in [23,25]. Typically, between 11–22 μg/ml of SERCA was added to a buffer containing 40 mM Hepes/KOH (pH 7.2 or pH 6.0), 5 mM $MgSO_4$, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, 8.0 units of pyruvate kinase, 20 units of lactate dehydrogenase, 1.01 mM EGTA and 2.1 mM ATP. In all experiments, unless otherwise stated, a free Ca^{2+} concentration of 6 μM (pCa 5.2) was used. Free Ca^{2+} concentrations were calculated as described in [26].

Phosphorylation studies

Phosphorylation of SERCA by [γ-^{32}P]ATP was carried out at 25 °C as described in [4,7,8]. The SERCA was diluted to 0.1 mg/ml in 20 mM Hepes/Tris (pH 7.2) containing 100 mM KCl, 5 mM $MgSO_4$ and 1 mM $CaCl_2$ in a total volume of 1 ml. Stocks of ATP (0.5 mM and 5 mM) with specific radioactivities of 100 Ci/mol or 10 Ci/mol respectively were made in the above buffer to cover the range between 0 and 25 μM. The reaction was started by the addition of [γ-^{32}P]ATP and stopped by the addition of ice-cold 40 % (w/v) TCA (trichloroacetic acid) after 15 s. The assay was then placed on ice for approx. 30 min prior to filtration through Whatman GF/C filters. The filters were washed with 30 ml of 12 % (w/v) TCA including 0.2 M H_3PO_4 and left to dry. The filters were placed in scintillant and counted.

TBBPA binding to SERCA

The binding of TBBPA to SERCA was measured by monitoring the ability of the BFR to quench the tryptophan fluorescence of the SERCA. The protein was diluted to 1 μM in a buffer containing 40 mM Hepes/KOH (pH 7.2 or 6.0), 1 mM EGTA and 100 mM NaCl. Fluorescence intensity was measured in a Perkin-Elmer LS50B Fluorescence Spectrophotometer at 25 °C (excitation 280 nm, emission 340 nm). In other experiments, some inhibitors of SERCA {i.e. thapsigargin, BHQ [2,5-di-(t-butyl)-1,4-hydroquinone] and 4-*n*-nonylphenol} were added separately after 1 μM TBBPA to assess whether they could displace TBBPA binding and therefore reverse TBBPA-induced fluorescence quenching.

The rate constants for TBBPA binding to the SERCA and to phospholipid bilayers were assessed by monitoring either the rate of tryptophan fluorescence quenching of the ATPase or of the membrane probe, PDA [12-(1-pyrene)dodecanoic acid] incorporated into phospholipid bilayers [27]. Rapid kinetic fluorescence measurements of TBBPA binding to the SERCA were performed using a stopped-flow fluorescence spectrophotometer (Applied Photophysics, model SX17 MV). Briefly, the sample handling unit possesses two syringes, A and B (drive ratio 10:1), which are driven by a pneumatic ram. Tryptophan fluorescence quenching of the protein was monitored at 25 °C when 1 μM SERCA (in 20 mM Hepes, 100 mM KCl, 5 mM $MgSO_4$, pH 7.2), in syringe A, was rapidly mixed with 10 μM (final concentration) of TBBPA in DMSO, in syringe B, exciting at 280 nm and measuring the emission above 320 nm using a cut-off filter. In order to assess the binding of TBBPA to phospholipid membranes, unilamellar liposomes of DOPC (dioleylphosphatidylcholine) containing PDA at a molar ratio of 500:1 were prepared in the Hepes buffer as above, followed by probe sonication and a freeze–thaw cycle. The time course of TBBPA quenching of PDA within DOPC liposomes was measured using a Hi-Tech SFA-12 stopped-flow accessory attached to a PerkinElmer LS50B fluorimeter by mixing 90 μM liposomes (equivalent concentration of lipids found associated with 1 μM SERCA within the SR vesicles) with 10 μM TBBPA. Excitation was at 342 nm and emission at 395 nm.

Intrinsic tryptophan fluorescence measurements to monitor Ca^{2+}-induced conformational changes of the SERCA

The conformational change induced by the addition of Ca^{2+} to the SERCA was monitored by observing the change in intrinsic tryptophan fluorescence as described in [4,7,8]. SERCA (50 μg/ml in the absence and presence of 3.5 μM TBBPA) was added to a buffer containing 20 mM Hepes/Tris, 100 mM $MgSO_4$ (pH 7.0) and any conformational changes were measured as a percentage change in total tryptophan fluorescence over a range of free Ca^{2+} concentrations (10–100 μM) at 25 °C (excitation 285 nm, emission 325 nm).

Measurement of the transient kinetics of conformational changes associated with Ca^{2+}-binding and dissociation

Rapid kinetic fluorescence measurements were performed using a stopped-flow fluorescence spectrophotometer as described in [7,8]. In the Ca^{2+}-binding experiments, syringe A, containing the SERCA (1 μM) in 20 mM Hepes/Tris (pH 7.2), 100 mM KCl, 5 mM $MgSO_4$ and 50 μM EGTA, was rapidly mixed with syringe B, containing 1 mM Ca^{2+} (final concentration). In Ca^{2+} dissociation experiments, syringe A containing the SERCA (1 μM) in 20 mM Hepes/Tris (pH 7.2), 100 mM KCl, 5 mM $MgSO_4$ and 100 μM Ca^{2+} was rapidly mixed with syringe B containing 2 mM EGTA (final concentration). Tryptophan fluorescence was monitored at 25 °C by exciting the SERCA (rapidly mixed with buffers containing either Ca^{2+} or EGTA) at 280 nm and measuring the emission above 320 nm using a cut-off filter.

Molecular modelling

The program Molegro Virtual Docker 2007 (with the standard default settings) was used in order to predict the ligand–protein interactions of the energy-minimized conformational structure of TBBPA to the SERCA. The atomic co-ordinates for the E2 (thapsigargin plus BHQ) low-affinity Ca^{2+}-binding form of the SERCA (PDB code 2AGV) was used for this virtual docking procedure. Figures were produced using the Swiss PDB Viewer.

RESULTS

Figure 1 shows that TBBPA is a potent inhibitor of the skeletal muscle SERCA 1a isoform and that this inhibition is pH-sensitive. At pH 7.2 the apparent inhibition constant [K_i (app): concentration causing 50 % inhibition under these experimental conditions] was

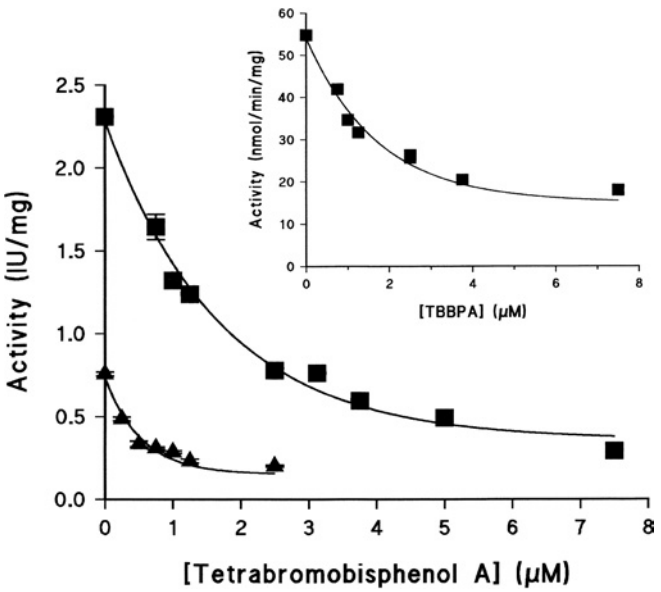

Figure 1 The effects of TBBPA on SERCA activity

SERCA activity at a range of TBBPA concentrations was measured in rabbit skeletal muscle SR membranes at 25 °C in a buffer at pH 7.2 (■) or pH 6.0 (▲). Inset, the effects of TBBPA on SERCA activity in cerebellar microsomes measured at pH 7.2 and 37 °C. The results represent the mean ± S.D. of 3–6 experiments.

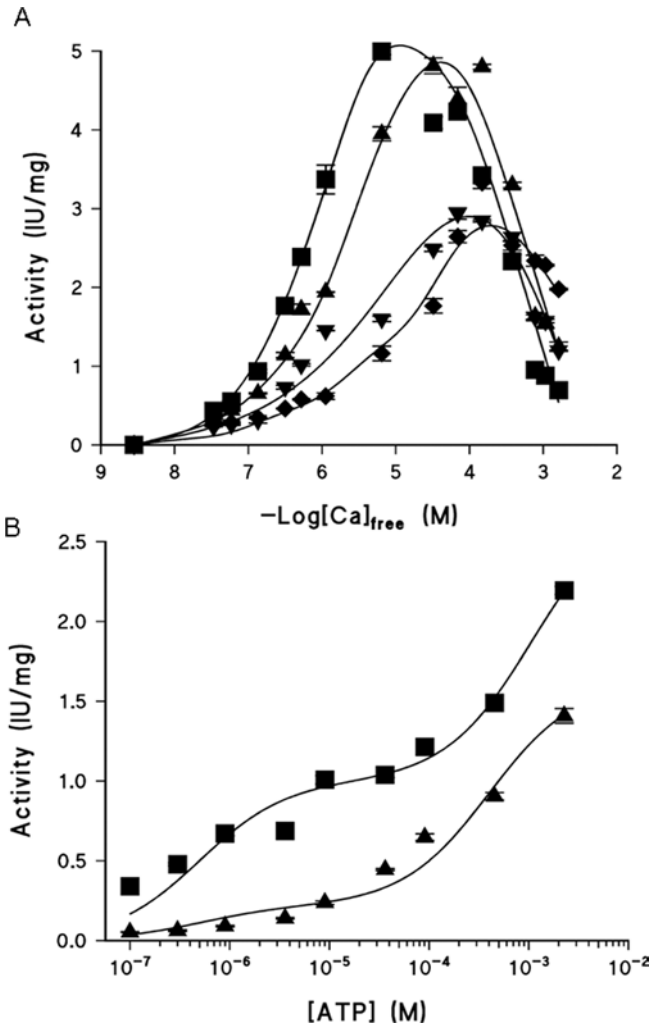

Figure 2 The effects of TBBPA on SERCA activity as a function of Ca^{2+} and ATP concentration

(**A**) The effects of increasing TBBPA and free Ca^{2+} concentration on SERCA activity measured at pH 7.2 and 25 °C. The concentrations of TBBPA used were 0 μM (■), 1 μM (▲), 2 μM (▼) and 4 μM (◆). (**B**) The effects of increasing ATP concentration on SERCA activity measured at pH 7.2 and 25 °C, in the absence (■) and the presence (▲) of 1.5 μM TBBPA. The lines represent the best fits to the results assuming a bi-Michaelis–Menten equation, with the parameters given in the text. The results represent the mean ± S.D. of 3–6 experiments.

determined to be 1.7 ± 0.3 μM, whereas at pH 6.0 the potency increased significantly [K_i (app)=0.46 ± 0.05 μM]. As low pH favours the E2 state of the SERCA [28], this might indicate that TBBPA preferentially binds to and stabilizes the ATPase in this conformation. This pH effect is, however, unlikely to be due to protonation of the phenol groups on TBBPA, since the pK_a for TBBPA was determined to be 9.8 ± 0.3 (typical of other phenol groups), which would indicate that TBBPA is essentially fully protonated at both pH 6 and pH 7.2. The cerebellum is known to express both the SERCA 2b and the SERCA 3 isoforms, and Figure 1 (inset) shows that TBBPA can also inhibit these isoforms, since it inhibits the cerebellar microsomal Ca^{2+}-dependent ATPase activity with an K_i (app) of 2.33 ± 0.45 μM at pH 7.2 (i.e. similar to that determined for SERCA 1a). Therefore it would seem that TBBPA is a non-selective SERCA isoform inhibitor, unlike some other inhibitors previously examined [9]. Complete inhibition, in all cases, was observed at or below 10 μM TBBPA. In comparison, we have also shown that the related, non-brominated compound, bisphenol A, a known endocrine disrupter which is used as a plasticizing agent, is a much weaker inhibitor of SERCA activity with a K_i of 233 ± 30 μM at pH 7.2 [6,29].

Figure 2(A) shows the effect of TBBPA on SERCA activity as a function of free Ca^{2+} concentration. The SERCA activity exhibited a classical 'bell-shaped' profile with respect to free Ca^{2+} concentration in the absence and presence of TBBPA. The stimulatory phase is associated with Ca^{2+} binding to the SERCA in an E1 (high affinity) form, increasing turnover rate, whereas the inhibitory phase is associated in part with the binding of Ca^{2+} to the E2 (low affinity) form of the enzyme. In the absence of TBBPA, the results gave a V_{max} of 5.0 ± 0.2 units/mg, with a K_m value of 0.62 ± 0.06 μM for the stimulatory phase and a K_m value of 0.62 ± 0.06 mM for the inhibitory phase. However, in the presence of TBBPA (between 1–4 μM), the results showed both a decrease in the maximal activity and an increase in the K_m for the stimulatory phase. At 1 μM TBBPA, the V_{max} was slightly decreased to 4.81 ± 0.40 units/mg, whereas the K_m values for the stimulatory and inhibitory phases increased to 1.04 ± 0.1 μM and 0.68 ± 0.07 mM respectively. The changes became more apparent at 2.0 μM TBBPA, as the V_{max} decreased to 2.94 ± 0.15 units/mg, whereas the K_m values increased further to 1.96 ± 0.15 μM and > 1 mM for the stimulatory and inhibitory phases respectively. Furthermore, in the presence of 4 μM TBBPA, V_{max} decreased to 3.33 ± 0.25 units/mg, whereas both K_m values increased significantly (11.0 ± 0.10 μM for the stimulatory and > 1 mM for the inhibitory). These results suggest that TBBPA may be decreasing the binding affinity of the SERCA for Ca^{2+}.

Figure 2(B) shows the activity of SERCA at various ATP concentrations in the absence and presence of 1.5 μM TBBPA. The complex biphasic profiles have previously been modelled assuming a bi-Michaelis–Menten process described in [5,8,30], where it is assumed that two independent ATP-binding events occur during enzymatic turnover, designated as the high-affinity catalytic site and the lower-affinity regulatory site. ATP binds and phosphorylates the SERCA at the high-affinity catalytic site,

whereas the low affinity regulatory site accelerates the cycle rate of the ATPase. In the absence of TBBPA, the results could be fitted assuming a catalytic K_m and V_{max} of 0.5 μM and 1.0 units/mg respectively; and a regulatory K_m and V_{max} of 1.0 mM and 1.8 units/mg respectively. In the presence of 1.5 μM TBBPA, only the catalytic V_{max} appeared substantially different compared with control, whereas all other parameters showed little or no differences (i.e. catalytic $K_m = 0.5$ μM, catalytic $V_{max} = 0.2$ units/mg; regulatory $K_m = 0.4$ mM, regulatory $V_{max} = 1.4$ units/mg).

In order to assess the possible effect of TBBPA on the phosphorylation of SERCA by ATP, experiments were performed at the optimal ATP concentration (25 μM) and varied concentrations of TBBPA. As shown in Figure 3(A), the level of SERCA phosphorylation was reduced in the presence of TBBPA, with a K_i of 2.0 ± 0.5 μM. As changes in the affinity of ATP binding to the SERCA can alter the level of phosphorylation by affecting the K_{ATP} [concentration of ATP required to achieve 50 % of the E-P_{max} (maximal level of enzyme phosphorylation)], the effects of ATP concentration in the absence and presence of 2.5 μM TBBPA were further investigated (Figure 3B). As shown in Figure 3(B), E-P_{max} and K_{ATP} values of 9.9 ± 0.9 nmol/mg and 0.9 ± 0.5 μM respectively were obtained for the control, whereas in the presence of 2.5 μM TBBPA, E-P_{max} and K_{ATP} were 6.7 ± 0.6 nmol/mg and 2.3 ± 0.8 μM respectively. Thus TBBPA caused a decrease in the maximum phosphorylation level of the SERCA (Figure 3A), but only had modest effects on the ATP affinity (i.e. K_{ATP}).

Figure 4(A) shows the binding of TBBPA to SERCA monitored by tryptophan fluorescence quenching at pH 7.2 and pH 6.0. Both sets of results could be reasonably fitted using a simple one-site binding relationship where the K_d values were determined to be 16 μM and 27 μM for pH 6 and pH 7.2 respectively (statistical analysis of goodness-of-fit as assessed by χ^2 tests was determined to be around 0.97, shown by the dotted lines). Slightly better fits to the results could, however, be achieved assuming a two-site binding relationship ($\chi^2 \geqslant 0.992$). Therefore our results would favour the possibility that fluorescence quenching of the SERCA by TBBPA could be best represented assuming high- and low-affinity binding sites. The continuous lines presented in Figure 4(A) for the pH 6.0 results were achieved assuming K_d values of 1.0 μM and 19 μM, whereas the fit to the pH 7.2 results was achieved assuming K_d values for the two sites of 2.2 μM and 44 μM for the high- and low-affinity sites respectively. These results would therefore indicate that the high-affinity K_d values obtained here probably relate to the binding sites on the SERCA that are associated with inhibition, since they are comparable with the K_i values. The fluorescence quenching of the SERCA at low concentrations of TBBPA (1 μM) was used to determine whether other commonly used hydrophobic inhibitors competed for the same site on the SERCA as TBBPA. This was achieved by determining whether the quenching caused by 1 μM TBBPA could be reversed by displacing it from its binding site. Figure 4(B) shows fluorescence traces of TBBPA quenching followed by the addition of a variety of inhibitors. It can be clearly seen that thapsigargin (two additions of 1 μM) showed no reversibility of the TBBPA quenching, however both BHQ (two additions of 5 μM) and 4-*n*-nonylphenol (two additions of 5 μM) inhibitors could significantly reverse TBBPA quenching upon their addition, indicating competitive binding. The displacement of TBBPA by CPA (cyclopiazonic acid), another commonly used SERCA inhibitor, was also assessed; but as CPA caused substantial tryptophan fluorescence quenching itself, its ability to displace TBBPA from the SERCA could not be determined by this method. As a control, Figure 4(B) also shows the effects of these inhibitors on the SERCA tryptophan fluoresence in the absence of TBBPA.

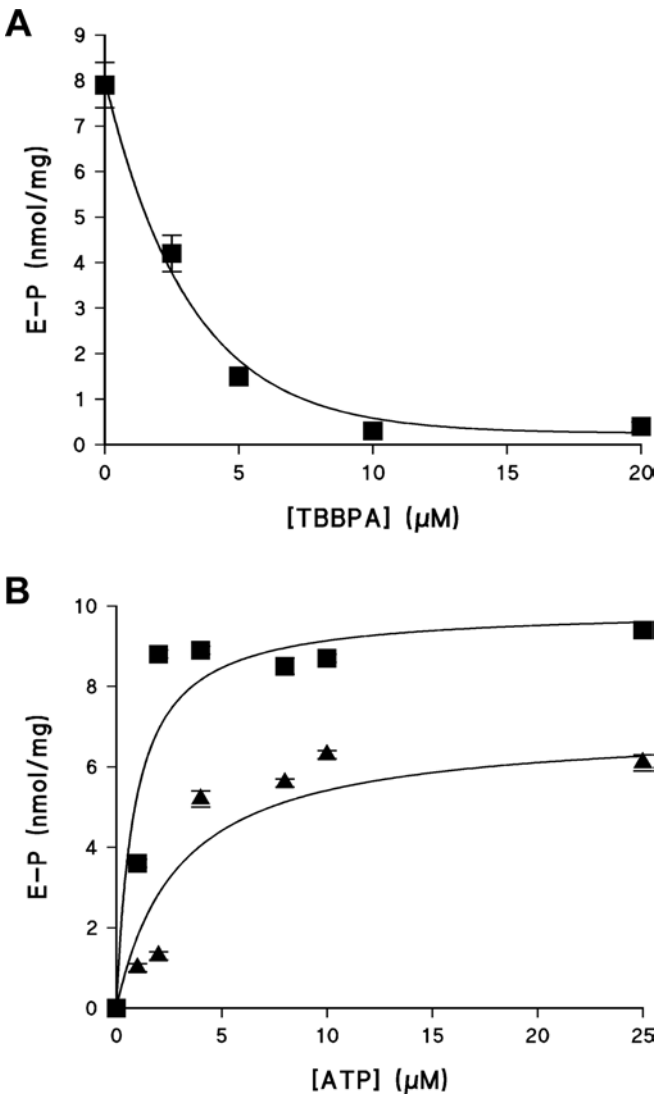

Figure 3 Effects of TBBPA on phosphorylation of SERCA by ATP

(**A**) The effect of increasing TBBPA concentration (0–20 μM) on the phosphorylation of SERCA in the presence of Ca^{2+} (1 mM) by [γ-^{32}P]ATP (25 μM), at pH 7.2 and 25 °C. (**B**) ATP-dependent phosphorylation of the SERCA over a range of [γ-^{32}P]ATP concentrations (0–25 μM) is affected in the absence (■) and presence (▲) of 2.5 μM TBBPA. The results represent the mean ± S.D. of 3 experiments.

As can be seen these inhibitors do not themselves cause an increase in tryptophan fluorescence of the SERCA in the absence of TBBPA, although BHQ and TG did cause a slight (1–2 %) quenching in fluorescence.

In order to assess whether TBBPA binds directly to the SERCA or indirectly via the phospholipid bilayer, the rate of TBBPA binding to the ATPase and to DOPC unilamellar liposomes (at the same lipid concentration found within the SR vesicles) was investigated. Figure 5(A) shows the rate of quenching of tryptophan fluorescence within the SERCA. Quenching was complete within less than 50 ms and the quenching process could be fitted to a simple first-order decay process with a rate constant of 90 s^{-1}. The rate of TBBPA quenching of PDA within the DOPC membranes was considerably slower, i.e. requiring about 10 s for completion (rate constant 0.24 s^{-1}; Figure 5B). These results would suggest that TBBPA binds directly to the SERCA, without the need to partition first into the phospholipid bilayer.

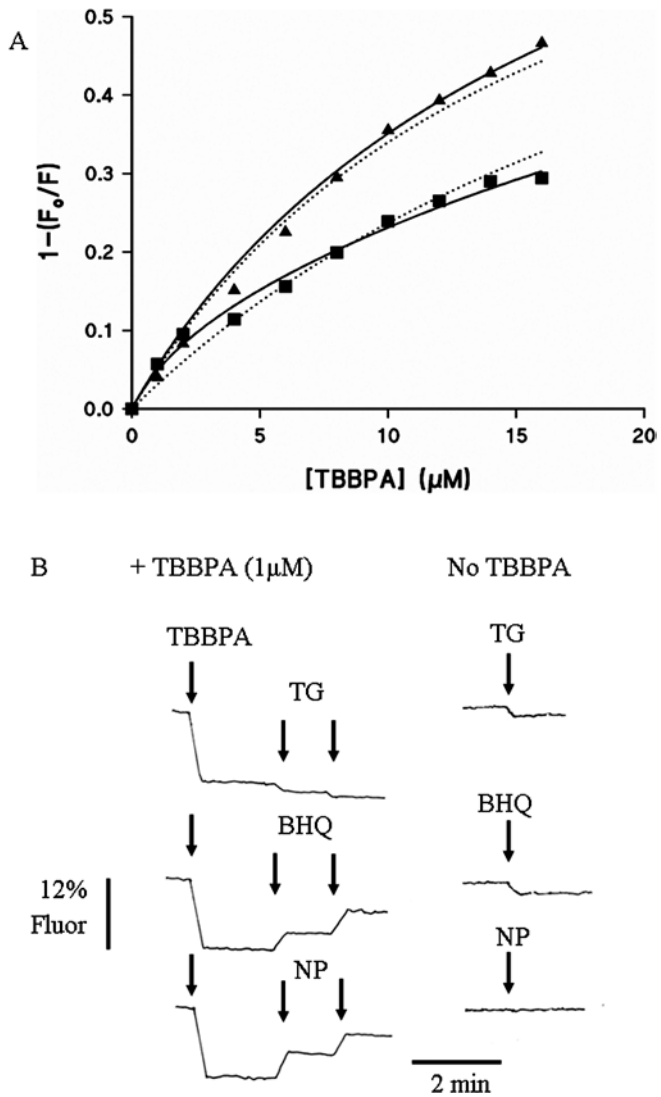

Figure 4 The quenching effects of TBBPA on the tryptophan fluorescence of SERCA

(**A**) The effects of TBBPA on the tryptophan fluorescence of SERCA measured at pH 7.2 (■) or pH 6.0 (▲) at 25 °C. The continuous lines represent the best fit of the results obtained assuming two-site binding (parameters given in text), whereas the dotted lines represent the best fit of the results obtained assuming one-site binding (parameters given in text). The results represent the mean values ± S.D. of 3 experiments. (**B**) Traces of tryptophan fluorescence levels upon quenching by the addition of 1 μM TBBPA. Following this, two additions of thapsigargin (TG, 1 μM each) were made. In subsequent experiments, the quenching of 1 μM TBBPA was reversed by either two additions of BHQ (5 μM each), or of 4-*n*-nonylphenol (NP, 5 μM each). Control traces in which thapsigargin (TG, 1 μM), BHQ (5 μM) and 4-*n*-nonylphenol (NP, 5 μM) were added in the absence of TBBPA are also shown. Each trace is representative of 3 identical experiments.

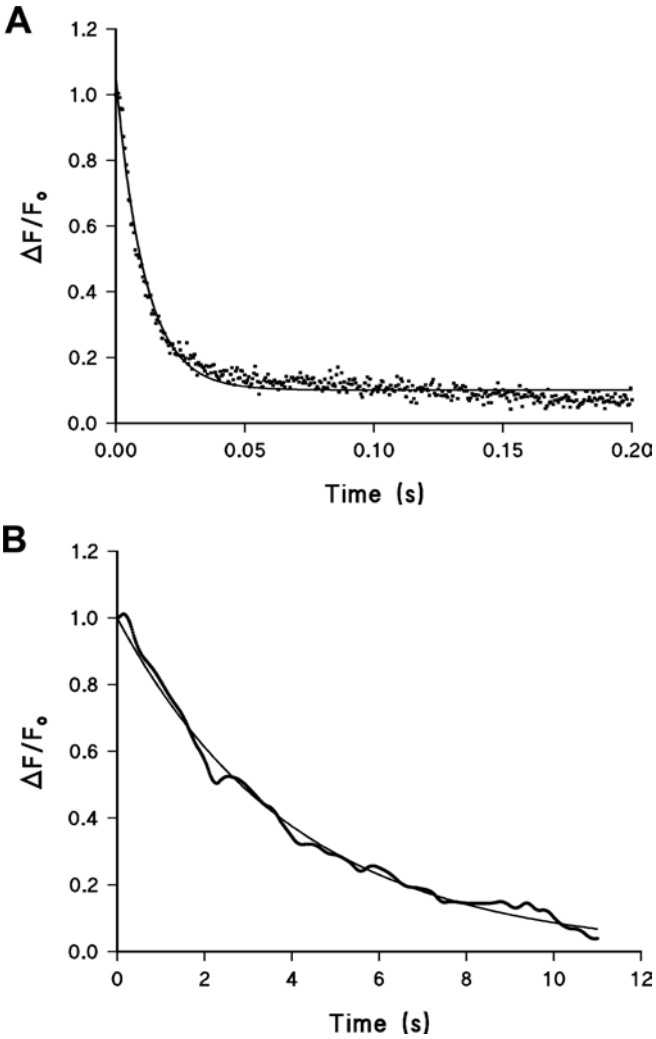

Figure 5 Kinetics of TBBPA binding to the SERCA and to phospholipid bilayers

(**A**) The rate at which the tryptophan fluorescence of SERCA (1 μM final concentration, which is equivalent to 90 μM phospholipid) is quenched by the addition of TBBPA (10 μM, final concentration), at pH 7.2 and 25 °C. The continuous line represents the best fit to the experimental results assuming a first-order decay process ($k_{obs} = 90\ s^{-1}$). The graph represents the mean of 8 accumulated runs. (**B**) The rate of TBBPA (10 μM) quenching of the membrane-bound PDA probe within DOPC unilamellar liposomes (90 μM final concentration). The fitted line represents the best fit to the experimental results, assuming a first-order decay process ($k_{obs} = 0.24\ s^{-1}$).

Figure 6(A) shows the effect of 3.5 μM TBBPA on the tryptophan fluorescence changes of SERCA upon the addition of a range of free Ca^{2+} concentrations, which is often used to assess the affinity of Ca^{2+} binding to the ATPase [7,8,31]. In the absence of TBBPA, the addition of Ca^{2+} gave a ΔF_{max} of 9.1 ± 0.3 % and a K_d (app) of 0.7 ± 0.1 μM, whereas in the presence of 3.5 μM TBBPA, there was a decrease in the maximal fluorescence change ($\Delta F_{max} = 5.21 \pm 0.04$ %) and a substantial increase in K_d (app). This results also suggests that TBBPA decreases the affinity of the SERCA for Ca^{2+}. Figure 6(B) illustrates the effect of varying TBBPA concentration on tryptophan fluorescence changes of the SERCA upon the addition of Ca^{2+} at pH 7.2. In the absence of TBBPA, addition of Ca^{2+} to the SERCA caused a 9.09 ± 0.31 % decrease in tryptophan fluorescence, whereas in the presence of 10 μM TBBPA, a 3.76 ± 0.66 % decrease in tryptophan fluorescence resulted. The IC_{50} for TBBPA under these conditions is about 3.5 μM. It is also apparent from the results in Figure 6(A) that there is also a small increase in fluorescence over the 10 nM to 10 μM free Ca^{2+} range in the presence of 3.5 μM TBBPA. However, since under these experimental conditions only a proportion of the SERCA is affected by TBBPA (Figure 6B), this would suggest that this component of the fluorescence change is due to the proportion of SERCA unaffected by TBBPA [thus having an K_d (app) similar to control].

At pH 7.2, the rate constants for the conformational changes associated with either Ca^{2+} binding to, or its dissociation from, the SERCA were measured in the absence and presence of TBBPA following the Ca^{2+}-induced changes in tryptophan fluorescence

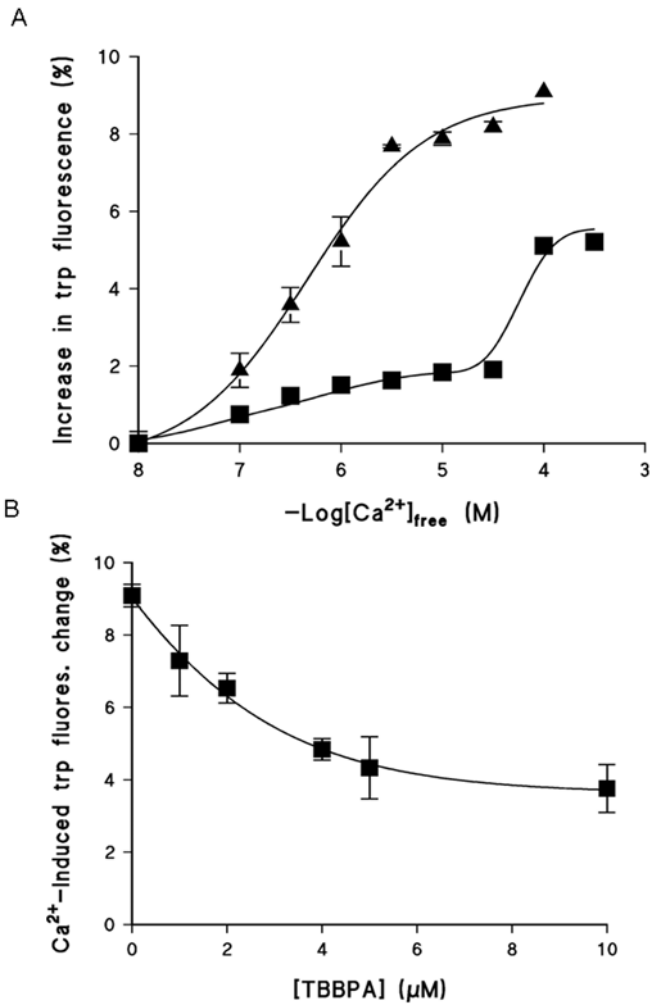

Figure 6 The effects of TBBPA on the Ca^{2+}-induced tryptophan fluorescence change of the SERCA

(A) The changes in SERCA tryptophan fluorescence induced by increasing free Ca^{2+} concentration in the absence (▲) or presence (■) of 3.5 μM TBBPA measured at pH 7.0 and 25 °C. (**B**) The effect of varied TBBPA concentration on the maximal tryptophan fluorescence change induced by the addition of 100 μM free Ca^{2+}. The results shown are the mean ± S.D. of at least 3 experiments.

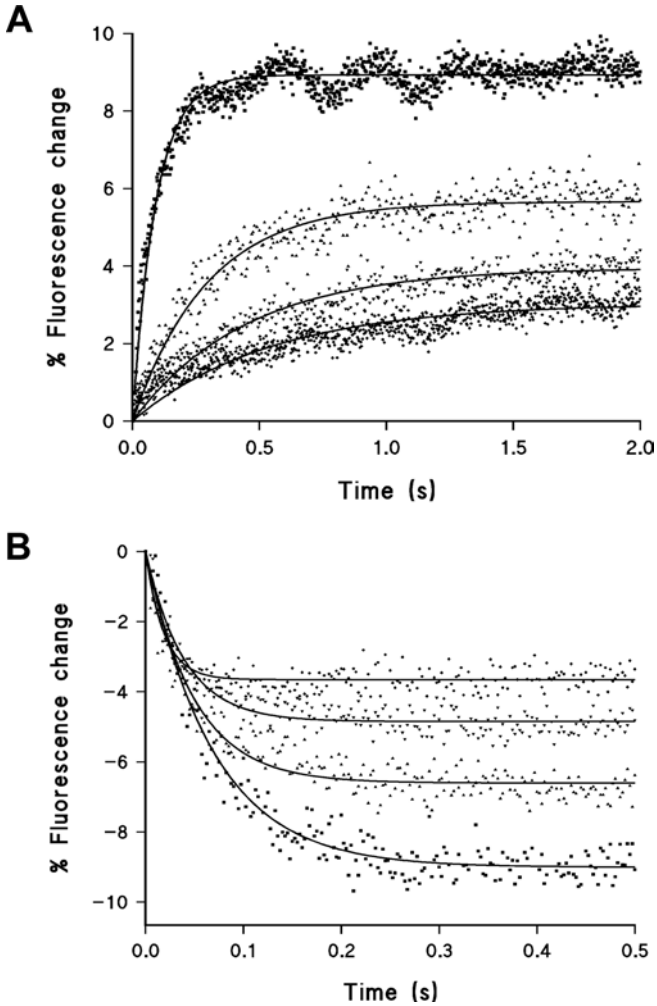

Figure 7 The effects of TBBPA on the kinetics of the Ca^{2+}-induced tryptophan fluorescence change of the SERCA

(**A**) The effect of increasing TBBPA concentrations (traces, from top to bottom, of 0, 2, 4 and 10 μM) on Ca^{2+}-induced conformational change monitored by following the increase in tryptophan fluorescence of the SERCA upon addition of Ca^{2+}. (**B**) The effects of increasing concentrations of TBBPA (from bottom to top, 0, 2, 4 and 10 μM) on the conformational change following Ca^{2+} dissociation from SERCA, monitored as a decrease in tryptophan fluorescence. The traces are the means of 8–10 accumulated runs and the continous lines are the best fits to the experimental results assuming a first-order process, with the parameters given in Table 1. The wavy appearances of some of the traces are due to the over-sampling function on the stopped-flow being employed to reduce the signal-to-noise levels.

using stopped-flow spectrofluorimetry. In Figures 7(A) and 7(B), all the results could be fitted to a mono-exponential equation (rate constants given in Table 1), as this is the simplest relationship which gave good fits to the results obtained in these experiments. As shown in Table 1, the rate constant for Ca^{2+} binding decreased, whereas the rate constant for Ca^{2+} dissociation increased with increasing TBBPA concentration. Consequently, the K_d for Ca^{2+} binding (calculated from the on and off rate constants) increased proportionally with increasing TBBPA concentration from 1.4 μM in the absence of TBBPA to approx. 30 μM in the presence of 10 μM TBBPA (Table 1).

Table 1 The kinetic parameters of Ca^{2+} binding and dissociation from the SERCA in the presences of TBBPA

Values are presented as mean ± S.E.M. of an average of 8–10 individual experiments.

TBBPA (μM)	k_{on} ($\mu M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	K_d (k_{off}/k_{on}) (μM)
0	10.2 ± 0.1	14.5 ± 0.4	1.4
2	3.4 ± 0.1	20.4 ± 0.7	6.0
4	2.2 ± 0.1	25.5 ± 1.0	11.6
10	1.8 ± 0.1	53.6 ± 3.4	29.8

DISCUSSION

BFRs are becoming prevalent throughout our environment, and many of these chemicals have been shown to bio-accumulate within animals and man to substantial levels [11–15]. Some studies have shown that in some individuals, levels over 1000 ng/g of lipid in human breast milk have been found [32]. Toxicological studies have shown that a number of these BFRs are both neurotoxic and can act as endocrine disrupters at the cellular level [20,21,33] and that TBBPA can disturb Ca^{2+} signalling pathways at low micromolar concentrations [20,22]. One obvious

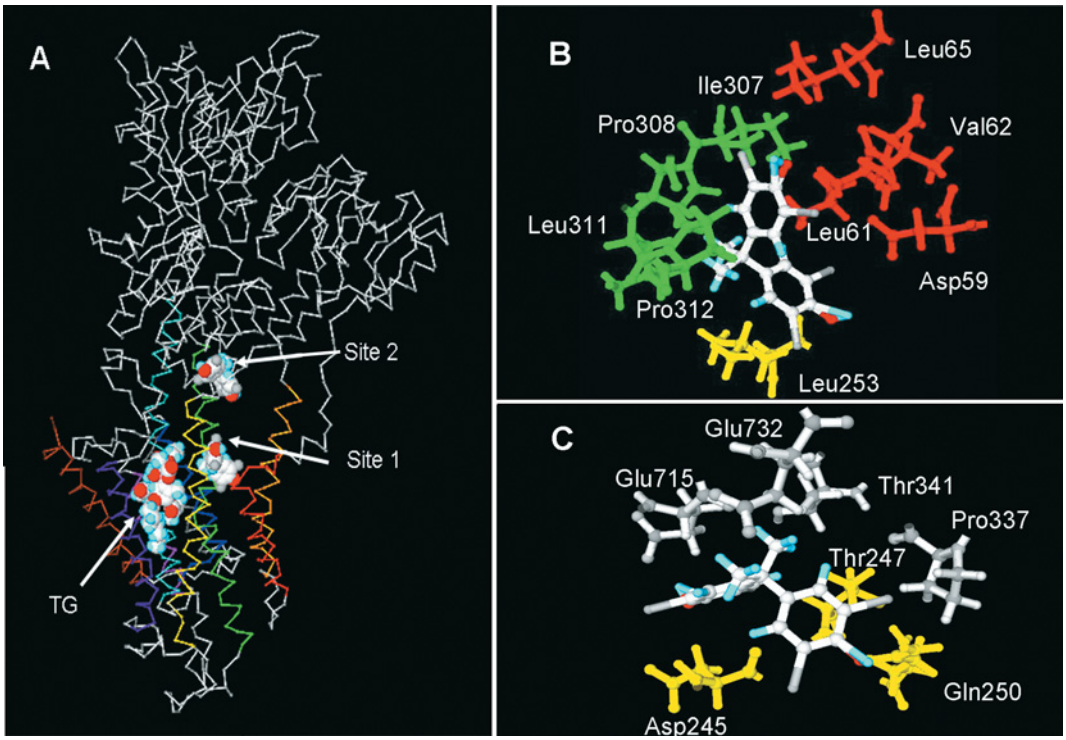

Figure 8 Predicted binding sites for TBBPA

The Molegro Virtual Docker 2007 was used to predict potential TBBPA binding sites on the E2 (thapsigargin and BHQ) SERCA 1a crystal structure (PBD code 2AGV). (**A**) The two potential TBBPA binding sites (labelled site 1 and site 2). In addition, the thapsigargin (TG) binding site is also shown. The transmembrane helices are colour coded accordingly: M1 (red), M2 (orange), M3 (yellow), M4 (green), M5 (cyan), M6 (blue), M7 (purple), M8 (pink), M9 (dark grey, behind thapsigargin-binding site), M10 (brown). (**B**) A close up of TBBPA predicted binding site 1, specifically highlighting amino acids within 4 Å (1 Å = 0.1 nm) of TBBPA and therefore able to potentially form either hydrogen bonds or Van der Waals interactions. (**C**) A close-up of the predicted TBBPA binding site 2.

possibility for this is that TBBPA can modulate the activity of Ca^{2+} transporters such as SERCA Ca^{2+} pumps.

Based on the SERCA activity results from the present study, TBBPA proved to be a very potent inhibitor of the ATPase, and affects the SERCA isoform 1a, and the isoforms 2b and 3 found in cerebellar microsomes, to similar extents. The inhibition of SERCA activity by TBBPA is also pH-sensitive, with a low pH favouring increased inhibition. The fact that under optimal conditions the K_i for inhibition is only 2–3 times higher than the molarity of the SERCA in these assays would likely indicate that the SERCA has one (or only a few) binding site(s) for TBBPA which, when occupied, inhibits activity. It is also clear that there is likely to be a number of sites of differing affinities for TBBPA on the SERCA as determined from fluorescence quenching studies, however only occupation of the high-affinity site(s) is/are required to elicit enzyme inhibition. These sites are also likely to be accessible directly from the aqueous phase rather than via the lipid bilayer.

It is unlikely that the major factor causing inhibition is due to TBBPA effects on ATP binding or phosphoryl-transfer, from the results obtained in the present study. However, what does appear to be the major factor for inhibition is the observation that TBBPA dramatically reduces the affinity of the SERCA for Ca^{2+} binding, and this reduction is due to both a decrease in the rate of binding and an increase in the rate of Ca^{2+} dissociation from the ATPase. The calculated affinity for Ca^{2+} binding was shown to be dramatically reduced from 1.4 μM to 30 μM in the presence of TBBPA.

Binding site competition studies, utilizing the displacement of TBBPA (which quenches tryptophan fluorescence of the SERCA) with a variety of other SERCA inhibitors have shown that the quenching could be substantially reversed by BHQ and 4-*n*-nonylphenol, both of which have been shown to stabilize the ATPase in an E2 conformational state [4,10]. The fact that TBBPA is a more potent inhibitor at low pH (which favours the E2 form [28]), in addition to TBBPA decreasing the Ca^{2+} affinity of SERCA (again consistent with being in an E2 state), would lead us to propose that TBBPA also inhibits the ATPase by stabilizing it in the E2 conformation. However, as TBBPA quenching of the SERCA is not reversed by thapsigargin (which does stabilize the E2 form [10]), this indicates that the site occupied by thapsigargin is distinct from that of TBBPA. Indeed, recent crystallographic studies have shown that thapsigargin and BHQ do bind to distinct sites within the SERCA [34], with BHQ binding to a hydrophobic pocket of the E2 state within the transmembrane regions encompassed by M1, M2, M3 and M4. Using the high accuracy molecular docking program Molegro Virtual Docker 2007, two potential binding sites for TBBPA on the E2 form of the SERCA were predicted (Figure 8A). These two sites are distinct from the thapsigargin binding site and are both located within a large cavity encompassed by the transmembrane helices M1, M2, M3 and M4, and enclosed at the top of the pocket by regions of the phosphorylation domain. Figure 8(B) shows the predicted TBBPA binding site 1, which is in an identical position to where BHQ binds within the crystal structure [34], i.e. in a hydrophobic region at the bottom of the cavity encircled by: Asp^{59}, Leu^{61} and Val^{62} (within M1); Leu^{253} (within M3); and Pro^{308}, Leu^{311} and Pro^{312} (within M4). This region within M4 is close to the Ca^{2+} binding site II and could well explain the effect of TBBPA on the affinity for Ca^{2+} binding. Figure 8(C) shows details of the predicted TBBPA binding site 2 found towards the top of this cavity, consisting of the top of transmembrane helix M3

(Asp^{245}, Thr^{247} and Gln^{250}), the start of the phosphorylation domain at Thr^{341} and Pro^{337} (within helix P1), and at Glu^{715} and Glu^{732}, also within the phosphorylation domain (at the ends of strands P6 and P7 respectively). Binding at this site may account in part for the effect of TBBPA reducing ATP-dependent phosphorylation. Our experimental results probably favour site 1, because BHQ can reverse TBBPA quenching; however, they are not necessarily mutually exclusive, as TBBPA may conceivably bind to both sites. Although there are no tryptophan residues close to these binding sites, it seems clear that, since TBBPA can bind directly to the SERCA from the aqueous phase, it most likely enters into this cavity by passing close to the Trp^{107} (at the top of M2). As binding of TBBPA to the SERCA is dynamic at any given time within some ATPases, TBBPA must therefore be in close proximity to Trp^{107} for fluorescence quenching to take place.

Previously, this cavity has been implicated as being a Ca^{2+} entry pathway, allowing Ca^{2+} to enter into specific binding sites buried deep within the transmembrane region [35], and this is supported by our results that show that TBBPA reduces Ca^{2+} binding to the SERCA. However, it is interesting to note that activity, mutagenesis and modelling results obtained for 2-APB (2-aminoethoxydiphenyl borate), a low-affinity SERCA inhibitor, show a reduction in Ca^{2+} binding by affecting both Ca^{2+} association and dissociation [8]. We deduced, from that study, that 2-APB binds on the adjacent side of the transmembrane bundle of the SERCA protein within a hydrophobic pocket defined by M3, M5 and M6 [8], and also occluded the Ca^{2+} entry pathway. From the present study with TBBPA, we suggest that the cavity encompassed by M1, M2, M3 and M4 forms the pathway for Ca^{2+} entry, since it also reduces the affinity for Ca^{2+} binding by affecting both on and off rates for Ca^{2+} binding. As we know that there are two Ca^{2+} binding sites on SERCA and that a number of potential Ca^{2+} entry pathway sites have been proposed by several groups [36–40] as to the exact route for Ca^{2+} to enter and bind, it would be tempting to speculate that each Ca^{2+} ion enters their respective binding sites via a distinct pathway, located on adjacent sides of the transmembrane helix bundle. One piece of evidence which may support this view is, when the SERCA is reconstituted with short chain length phospholipids, it reduces the stochiometry for Ca^{2+} binding from 2 to 1 without significantly affecting its affinity or the phosphorylation level [41]. Thus this indicates that the two Ca^{2+} binding events can, under some circumstances, be made distinct.

In summary, one of the most commonly used BFRs, TBBPA, which has been shown to be cytotoxic and causes disturbances of intracellular Ca^{2+} homoeostasis within cells in culture, may elicit its effects, at least in part, by inhibiting the SERCA Ca^{2+} pumps.

Dr Jon Ride (School of Biosciences, University of Birmingham, Birmingham, U.K.) is thanked for helping with the Molegro virtual docking program. We thank Houdini Ho Tin Wu (School of Biosciences, University of Birmingham, U.K.) for preliminary experiments. The College of Medicine, University of Ibadan, Nigeria and the School of Biosciences, University of Birmingham, U.K. are thanked for partial scholarship support to O.A.O. A grant from the Medical Faculty of the University of Birmingham was also awarded to undertake this work.

REFERENCES

1 Berridge, M. J., Bootman, M. D. and Lipp, P. (1998) Calcium: a life and death signal. Nature **395**, 645–648
2 Toyoshima, C. and Inesi, G. (2004) Structural basis of ion pumping by Ca^{2+}-ATPase of the sarcoplasmic reticulum. Annu. Rev. Biochem. **73**, 269–292
3 Wu, K. D., Lee, W. S., Wey, J. D., Bungard, D. and Lytton, J. (1995) Localization and quantification of endoplasmic reticulum Ca $^{2+}$-ATPase isoform transcripts. Am. J. Physiol. **269**, C775–C784
4 Michelangeli, F., Orlowski, S., Champeil, P., East, J. M. and Lee, A. G. (1990) Mechanism of inhibition of the (Ca^{2+}-Mg^{2+})-ATPase by nonylphenol. Biochemistry **29**, 3091–3101
5 Brown, G. R., Benyon, S. L., Kirk, C. J., Wictome, M., East, J. M., Lee, A. G. and Michelangeli, F. (1994) Characterisation of a novel Ca^{2+} pump inhibitor (*bis*-phenol) and its effects on intracellular Ca^{2+} mobilization. Biochim. Biophys. Acta **1195**, 252–258
6 Hughes, P. J., McLellan, H., Lowes, D. A., Khan, S. Z., Bilmen, J. G., Tovey, S. C., Godfrey, R. E., Michell, R. H., Kirk, C. J. and Michelangeli, F. (2000) Estrogenic alkylphenols induce cell death by inhibiting testis endoplasmic reticulum Ca^{2+} pumps. Biochem. Biophys. Res. Commun. **277**, 568–574
7 Longland, C. L., Mezna, M. and Michelangeli, F. (1999) The mechanism of inhibition of the Ca^{2+}-ATPase by mastoparan. Mastoparan abolishes cooperative Ca^{2+} binding. J. Biol. Chem. **274**, 14799–14805
8 Bilmen, J. G., Wootton, L. L., Godfrey, R. E., Smart, O. S. and Michelangeli, F. (2002) Inhibition of SERCA Ca^{2+} pumps by 2-aminoethoxydiphenyl borate (2-APB). 2-APB reduces both Ca^{2+} binding and phosphoryl transfer from ATP, by interfering with the pathway leading to the Ca^{2+}-binding sites. Eur. J. Biochem. **269**, 3678–3687
9 Wootton, L. L. and Michelangeli, F. (2006) The effects of the phenylalanine 256 to valine mutation on the sensitivity of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) Ca^{2+} pump isoforms 1, 2, and 3 to thapsigargin and other inhibitors. J. Biol. Chem. **281**, 6970–6976
10 Wictome, M., Michelangeli, F., Lee, A. G. and East, J. M. (1992) The inhibitors thapsigargin and 2,5-di(tert-butyl)-1,4-benzohydroquinone favour the E2 form of the Ca^{2+}, Mg^{2+}-ATPase. FEBS Lett. **304**, 109–113
11 de Wit, C. A. (2002) An overview of brominated flame retardants in the environment. Chemosphere **46**, 583–624
12 Alaee, M. and Wenning, R. J. (2002) The significance of brominated flame retardants in the environment: current understanding, issues and challenges. Chemosphere **46**, 579–582
13 Law, R. J., Allchin, C. R., de Boer, J., Covaci, A., Herzke, D., Lepom, P., Morris, S., Tronczynski, J. and de Wit, C. A. (2006) Levels and trends of brominated flame retardants in the European environment. Chromosphere **64**, 187–208
14 Sjodin, A., Jones, R. S., Focant, J. F., Lapeza, C., Wang, R. Y., McGahee, E. E., Zhang, Y., Turner, W. E., Slazyk, B., Needham, L. L. and Patterson, D. G. (2004) Retrospective time-trend study of polybrominated diphenyl ether and polybrominated and polychlorinated biphenyl levels in human serum from the United States. Environ. Health Perspectives **12**, 654–658
15 Thomsen, C., Lundanes, E. and Becher, G. (2001) Brominated flame retardants in plasma samples from three different occupational groups in Norway. J. Environ. Monit. **3**, 366–370
16 Strack, S., Detzel, T., Wahl, M., Kuch, B. and Krug, H. F. (2007) Cytotoxicity of TBBPA and effects on proliferation, cell cycle and MAPK pathways in mammalian cells. Chemosphere **67**, S405–S411
17 Nakagawa, Y., Suzuki, T., Ishii, H. and Ogata, A. (2007) Biotransformation and cytotoxicity of a brominated flame retardant tetrabromobisphenol A and its analogues in rat hepatocytes. Xenobiotica **37**, 693–708
18 Pullen, S., Boecker, R. and Tiegs, G. (2003) The flame retardants tetrabromobisphenol A and tetrabromobisphenol A-bisallylether suppress the induction of interleukin-2 receptor alpha chain (CD25) in murine splenocytes. Toxicology **184**, 11–22
19 Fonnum, F., Mariussen, E and Reistad, T. (2006) Molecular mechanisms involved in the toxic effects of polychlorinated biphenyls and brominated flame retardants. J. Toxicol. Eviron. Health, Part A **69**, 21–35
20 Reistad, T., Mariussen, E., Ring, A. and Fonnum, F. (2007) *In vitro* toxicity of tetrabromobisphenol A on cerebellar granule cells: cell death, free radical formation, calcium influx and extracellular glutamate. Toxicol. Sci. **96**, 268–278
21 Kitamura, S., Jinno, N., Ohta, S., Kuroki, H. and Fujimoto, N. (2002) Thyroid activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. Biochem. Biophys. Res. Commun. **293**, 554–559
22 Reistad, T., Mariussen, E. and Fonnum, F. (2005) The effect of a brominated flame retardant, tetrabromobisphenol A, on free radical formation in human neutrophil granulocytes: the involvement of the MAP kinase pathway and protein kinase C. Toxicol. Sci. **83**, 89–100
23 Michelangeli, F. and Munkonge, F. M. (1991) Methods of reconstitution of the purified sarcoplasmic reticulum (Ca^{2+}-Mg^{2+})-ATPase using bile salt detergents to form membranes of defined lipid to protein ratios or sealed vesicles. Anal. Biochem. **194**, 231–236
24 Brown, G. R., Sayers, L. G., Kirk, C. J., Michell, R. H. and Michelangeli, F. (1992) The opening of the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channel in rat cerebellum is inhibited by caffeine. Biochem. J. **282**, 309–312
25 Longland, C. L., Mezna, M., Langel, U., Hallbrink, M., Soomets, U., Wheatley, M., Michelangeli, F. and Howl, J. (1998) Biochemical mechanisms of calcium mobilisation induced by mastoparan and chimeric hormone-mastoparan constructs. Cell Calcium **24**, 27–34

26 Gould, G. W., East, J. M., Froud, R. J., McWhirter, J. M., Stefanova, H. I. and Lee, A. G. (1986) A kinetic model for the Ca^{2+}-Mg^{2+}-activated ATPase of sarcoplasmic reticulum. Biochem. J. **237**, 217–227
27 Ogunbayo, O. A., Jensen, K. T. and Michelangeli, F. (2007) The interaction of the brominated flame retardant tetrabromobisphenol A with phospholipid membranes. Biochim. Biophys. Acta **1768**, 1559–1566
28 Michelangeli, F., Colyer, J., East, J. M. and Lee, A. G. (1990) Effect of pH on the activity of the Ca^{2+}-Mg^{2+}-activated ATPase of sarcoplasmic reticulum. Biochem. J. **267**, 423–429
29 Kirk, C. J., Bottomley, L., Minican, N., Carpenter, H., Shaw, S., Kohli, N., Winter, M., Taylor, E. W., Waring, R. H., Michelangeli, F. and Harris, R. M. (2003) Environmental endocrine disrupters dysregulate estrogen metabolism and Ca^{2+} homeostasis in fish and mammals via receptor-independent mechanisms. Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol. **135**, 1–8
30 Kosk-Kosicka, D., Kirzmack, M. and Inesi, G. (1983) Kinetic characterization of detergent-solubilized sarcoplasmic reticulum adenosinetriphosphatase. Biochemistry **22**, 2559–2567
31 Fernandez-Belda, F., Kurzmack, M. and Inesi, G. (1984) A comparative study of calcium transients by isotopic tracer, metallochromic indicator, and intrinsic fluorescence in sarcoplasmic reticulum ATPase. J. Biol. Chem. **259**, 9687–9698
32 Meironyte, D., Norn, K. and Bergman, A. (1999) Analysis of polybrominated diphenyl ethers in Swedish human milk. A time-related trend study, 1972–1997. J. Toxicol. Environ. Health, Part A **58**, 329–341
33 Meerts, I. A., Letcher, R. J., Hoving, S., Marsh, G., Bergman, A. and Lemmen, J. G. (2001) *In vitro* estrogenicity of polybrominated diphenyl ethers, hydroxylated PDBEs, and polybrominated bisphenol A compounds. Environ. Health Perspect. **109**, 399–407
34 Obara, K., Miyashita, N., Xu, C., Toyoshima, I., Sugita, Y., Inesi, G. and Toyoshima, C. (2005) Structural role of countertransport revealed in Ca^{2+} pump crystal structure in the absence of Ca^{2+}. Proc. Natl. Acad. Sci. U.S.A. **102**, 14489–14496
35 Moncoq, K., Trieber, C. A. and Young, H. S. (2007) The molecular basis for cyclopiazonic acid inhibition of the sarcoplasmic reticulum calcium pump. J. Biol. Chem. **282**, 9748–9757
36 Toyoshima, C., Nakasako, M., Nomura, H. and Ogawa, H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. Nature **405**, 647–655
37 Lee, A. G. and East, J. M. (2001) What the structure of a calcium pump tells us about its mechanism. Biochem. J. **356**, 665–683
38 Zhang, Z., Lewis, D., Sumbilla, C., Inesi, G. and Toyoshima, C. (2001) The role of the M6-M7 loop (L67) in stabilization of the phosphorylation and Ca^{2+} binding domains of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA). J. Biol. Chem. **276**, 15232–15239
39 Zhang, Z., Lewis, D., Strock, C., Inesi, G., Kakasako, M., Nomura, H. and Toyoshima, C. (2000) Detailed characterization of the cooperative mechanism of Ca^{2+} binding and catalytic activation in the Ca^{2+} transport (SERCA) ATPase. Biochemistry **39**, 8758–8767
40 Menguy, T., Corre, F., Bouneau, L., Deschamps, S., Møller, J. V., Champeil, P., le Maire, M. and Falson, P. (1998) The cytoplasmic loop located between transmembrane segments 6 and 7 controls activation by Ca^{2+} of sarcoplasmic reticulum Ca^{2+}-ATPase. J. Biol. Chem. **273**, 20134–20143
41 Michelangeli, F., Orlowski, S., Champeil, P., Grimes, E. A., East, J. M. and Lee, A. G. (1990) Effects of phospholipids on binding of calcium to Ca^{2+}-Mg^{2+}-ATPase. Biochemistry **29**, 8307–8312

Received 26 June 2007/15 August 2007; accepted 5 September 2007
Published as BJ Immediate Publication 5 September 2007, doi:10.1042/BJ20070843